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FLOW-SYSTEMS ANALYSIS AND CHARACTERIZATION OF PROTEIN CONTENTS AND PROLIFERATING KINETICS IN ASCITES AND SOLID TUMORS

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I. INTRODUCTION

Analysis of the cellular DNA and protein content in malignant tumors provides useful kinetic information for characterizing the dynamics of tumor cell growth and proliferation. Kinetic data have been used in the design of rational schedules for chemotherapy (1) and for determining the effects of various potential drug agents on cycle progression (2,3). However, obtaining cell kinetic information by conventional biochemical methods is often quite tedious and time-consuming. It would be extremely useful to have an analytical system available which could rapidly provide detailed information for characterization of tumors at various stages of development. Flow microfluorometery (FMF) has been shown to be a rapid and reliable approach for simultaneous determination of both DNA and protein in single-cell populations (4,5), thus providing information on both cell proliferation and cell growth. Since the methodology does not rely on cellular incorporation of ^{3}H-thymidine, the technique is especially useful for analysis of slowly traversing or arrested cells such as encountered in many tumor systems. Furthermore, the FMF system has the unique advantage of providing complete accountability of the proportion of cells at various stages in the cell cycle.

In this report, we present the initial results of our attempts to characterize the mouse L1210 ascites and Lewis lung carcinoma tumor systems using FMF analysis of cytochemically stained tumor cell populations. Time-sequence sampling and flow analysis were used to detect fluctuations in proliferation kinetics of tumor cell populations associated with tumor age and development. Other kinetic information relating to cell death and cell loss, as well as analysis of proliferating and nonproliferating compartments, obviously is necessary to characterize completely these model systems. However, FMF techniques, coupled with standard biochemical techniques, should provide additional detailed information for assaying the dynamics of tumor growth.

II. PROCEDURES AND MATERIALS USED

A. Tumor Systems

1. L1210 ASCITES

The L1210 leukemia was chosen as the standard model tumor reference

system for screening and evaluation of chemotherapeutic drugs during the 1974 World Conference on Drug Screening held in Geneva, Switzerland (6). The tumor originated in 1948 in the spleen and lymph nodes of mice whose skin had been painted with methylcholanthrene (7). The tumor is propagated in vivo in DBA/2 mice, and BDF_1 mice (C57Bl/6 x DBA/2) are commonly used for drug testing. In the present study, L1210 ascites were grown in DBA/2 mice following an initial intraperitoneal inoculum of 10^5 cells. Under these conditions, hemorrhaging was noticeable in the peritoneal cavity on about days six or seven and extensive at death (days eight to ten).

2. LEWIS LUNG CARCINOMA

The Lewis lung carcinoma (LLC) is a solid tumor system that has also been used for drug screening. Tumors are propagated as subcutaneous (sc) implants in C57Bl/6 mice, and tests are generally performed with BDF_1 mice serving as host animals. Primary (sc) tumors grow rapidly and eventually metastasize to the lung and other organs. This presents an interesting system for studying growth kinetics of the tumor under differing in vivo environmental conditions. In these studies, the LLC were grown initially as solid (sc) tumors in C57Bl/6 mice.

B. Cell Dispersal, Fixation, and Staining

DBA/2 mice were sacrificed at daily intervals beginning on day two after L1210 cell inoculation. Cells were harvested by aspiration, washed once in saline GM (balanced salt solution lacking calcium and magnesium) containing 0.5 mM EDTA, and fixed in 70% ethanol for subsequent DNA staining with mithramycin (100 µg/ml in normal saline containing 15 mM $MgCl_2$) (8). For staining both DNA and protein, L1210 cells were centrifuged from the ethanol fixative, resuspended in RNase (1 mg/ml, pH 7.0, Worthington, beef pancreatic RNase, code R), and incubated for 30 minutes at 37°C in a water bath. Following RNase hydrolysis, the cells were washed once in water and stained for total protein using fluorescein isothiocyanate (FITC in 0.5 M $NaHCO_3$ adjusted to pH 8.0, J. T. Baker), rinsed once in PBS, and then stained for DNA with propidium iodide (PI, CalBiochem, 0.1 mg/ml in PBS). Cells were rinsed in PBS and then resuspended in normal saline for analysis (5).

The solid Lewis lung carcinoma, primary and metastatic, as well as the spleen from nontumor-bearing mice, were forced through a 500-micron Teflon mesh into cold saline GM. The tumor material was pipetted 20-30 times through a 5-ml pipette and then filtered first through a 120-micron filter and then a 62-micron nylon filter to remove the larger tumor material. Cells were pelleted by centrifugation, washed once in a large volume of saline GM, and fixed in cold 70% ethanol. Cell staining for both DNA and protein was as described for L1210 ascites cells.

C. Flow-Systems Analysis

Simultaneous analysis of the DNA and protein contents of PI-FITC stained cells was performed using the multiparameter analysis and cell sorting system (9) previously described (5) using a 488-nm wavelength laser excitation source. The DNA content distribution of mithramycin-stained cells was obtained using a 457-nm laser line for excitation. Descriptions of the design and operational features of the single-parameter (10,11) and the multiparameter analysis and cell sorting systems (8,12) have been discussed elsewhere.

Cells were sorted from the 2C and 4C-8C regions of the LLC DNA distribution and analyzed microscopically as previously described (13). The relative proportions of cells in G_1, S, and G_2 + M were derived from the DNA distribution profiles using the Dean and Jett (14) computer program. Computer analysis was used to obtain the density contours for DNA-protein profiles of the L1210 ascites and the primary and lung metastases of the Lewis lung carcinoma.

III. RESULTS

A. Analysis of L1210 Ascites Cells

Typical DNA distribution patterns obtained for mithramycin-stained populations of L1210 cells obtained on days three, four, six, and seven following an inoculum of 10^5 cells are shown in Fig. 1. Comparison of these DNA distributions to those of normal (diploid) spleen cells (not shown) indicates that G_1 cells of L1210 have a 2C DNA content. The percentage of cells in G_1, S, and G_2 + M for days two through eight is provided in Table I. These data and data obtained from other experiments in our Laboratory clearly reveal changes in proliferation kinetic patterns that are concomitant with increased cell density or tumor age.

There is an initial lag in tumor growth as indicated by the low percentage of cells in S phase on day two; however, a rapid increase in cell proliferation is apparent by days four and five, followed by a precipitous decrease in proliferating cells on day six. This dramatic decrease in cell progression capacity could possibly be caused by release of cytotoxic substances during hemorrhaging which is quite apparent by day six. The percentage of cells in S phase remains unchanged on day seven; however, there is a significant increase in the G_2 + M fraction.

Simultaneous analysis of DNA and protein provides useful information relating to the biosynthetic capacity of cells at specific phases of the cell cycle (4,5). Since a gross imbalance in DNA/protein ratio will eventually lead to cell death, analysis of the quantitative relationship of these parameters can be useful for elucidating the occurrence of ensuing phase-specific cell death. Figure 2 shows the single-parameter and two-parameter DNA-protein profiles for L1210 ascites cells. The protein content distribution is similar to that obtained for cultured L1210 cells (not shown).

The DNA-protein contour profiles for L1210 ascites cells harvested on days three, four, six, and seven are shown in Fig. 3. Computer-generated density contour lines were obtained for arbitrary threshold settings of 20, 100, 200, 500, and 700 cells. These profiles reflect the same DNA distribution patterns (x axis) seen in Fig. 1; however, the protein distributions reveal a subpopulation of cells having a lower protein content than the bulk of the population. This subpopulation is most apparent on days three and four but is significantly decreased by days six and seven. Although these cells have not been sorted and morphologically identified at this time, it is speculated that, on the basis of cell volume studies performed in this Laboratory, this subpopulation of cells represents normal 2C diploid cells in the peritoneal cavity which are harvested along with tumor cells. These diploid cells initially represent a small but substantial proportion of the total cell population; however, as the tumor cell density increases rapidly, normal cells become less apparent. Figure 3 also

illustrates the rapid increase in proportion of cells having a large protein mass. All of the profiles show the accumulation of cell clumps which may be noted at the extreme right of each distribution.

B. Analysis of Primary and Lung Metastases of Lewis Lung Carcinoma

DNA and protein contour profiles of a primary tumor and lung metastases of the Lewis lung carcinoma from C57Bl/6 mice are shown in Fig. 4. Computer-generated density contours were obtained at threshold settings of 50, 100, 150, 200, 350, and 500 cells. Based on results obtained in both cell sorting experiments and DNA content analysis of diploid spleen cells, the portion of cells designated 2C in the DNA profile (x axis) shown in Fig. 4 represents normal cells and the remaining portion of the profile G_1 (4C), S, and G_2 + M (8C) populations of the tumor cells, respectively. The percentages of cells in G_1, S, and G_2 + M were 38.7, 54.9, and 6.4, respectively, for the primary tumor and 58.8, 32.0, and 9.2 for the metastatic population. Single-parameter DNA and protein distributions have been presented elsewhere (15).

It may be noticed that, in general, tumor cells have a greater protein mass than most but not all normal cells. Also, the protein profiles of the primary and lung metastases appear to be quite different, particularly through the S and G_2 + M regions. In particular, the primary tumor appears to contain cells having a wider range in protein content through these regions.

IV. DISCUSSION

The DNA distribution patterns obtained for L1210 ascites cells clearly reveal the fluctuations in cycle kinetic patterns associated with increased cell density or tumor age. Based on studies presented in this report, there is an initial lag phase reflected by the low percentage of cells in S phase on day two, followed by a rapid increase in cell proliferation (i.e., days four and five) and then a decrease in proliferation just prior to death of the host animal. The growth and proliferation patterns presented here are similar to those observed for L1210 cells in culture. In fact, the percentages of cells in G_1, S, and G_2 + M at the peak of growth on day five (Table I) are quite comparable to values obtained previously in our Laboratory for exponentially growing L1210 cells in vitro (i.e., percentages in G_1, S, and G_2 + M are 29.2, 65.5, and 8.3, respectively).

The DNA distribution patterns presented for L1210 ascites cells (Fig. 1) also contain the subpopulation of normal diploid cells revealed by the DNA-protein contour profiles (Fig. 2). Since normal cells have different ranges in protein content levels than tumor cells, it would be possible to strip the true DNA distribution of tumor cells from the normal cell population. For example, by gating on the protein content (green fluorescence) range of tumor cells, it is possible to analyze only the DNA (red fluorescence) of tumor cells. Analysis of the corrected DNA distributions would reflect more accurately the cycle distribution of cells than do the data in Table I, particularly for days two, three, and four where normal cells probably represent a small but significant proportion of the population. Gated analysis techniques are being employed in more recent studies.

Simpson-Herren et al. (16) have recently presented results of autoradiographic studies which demonstrate differences in proliferation of the primary and lung metastases of the Lewis lung carcinoma. The fraction of

cells in S phase for the lung metastases is in good agreement with the pulse-labeling index (36%) obtained by Simpson-Herren et al. (16) in spontaneous metastatic lung tumors 17 days post-implant of the primary (sc) tumors. However, these investigators found a much lower labeling index (25%) for primary tumors than the 54% S fraction obtained in our studies. Several significant differences in experimental design of both studies make it difficult to compare the results directly.

Differences observed in protein content levels of metastatic and primary Lewis lung carcinoma must await further studies for verification, since these represent only initial studies of this nature with this tumor system. It is possible that the metastatic tumor has certain characteristic properties, among which is a more rigid protein content range during specific portions of the cell cycle. On the other hand, the differences in protein content range may be caused by differences in the in vivo environment. This is to infer that differences in nutrient supply are available to the tumor in lung tissue, as opposed to the subcutaneous region of the mouse.

Normal cells are present in most solid tumors where they function in various aspects of nutrition (i.e., provide adequate blood supply) or aid in providing a framework system for tumor architecture. In spite of the fact that the numbers and types of normal cells must change during the various stages of tumor development, little attempt has been made to exploit this phenomenon for characterizing the developmental stages of tumor growth and proliferation. In the context of drug evaluation, the effects of various chemical agents on normal cells are often as important as the effects on the tumor cell population. In either instance, the use of techniques demonstrated here should be extremely useful. Cell sorting based on DNA content measurements can provide a concentrated population composed of large numbers of normal cells for microscopic analysis, while the protein content analyses of normal and tumor cells would be useful for determining the physiological condition of cells during drug testing experiments.

V. SUMMARY

Flow microfluorometric analysis of the DNA and protein content of mouse ascites and solid tumor cell populations grown in vivo has provided information on protein distribution of cells in various phases of the cell cycle, as well as age-associated changes in proliferation kinetics of these tumor systems. L1210 ascites cells and solid Lewis lung carcinomas were dispersed, fixed, and stained for both DNA and protein, respectively, using the fluorochromes propidium iodide (red) and fluorescein isothiocyanate (green). Simultaneous DNA and protein determinations were performed using a flow system in which rapid two-color analysis of stained cells was achieved at approximately 10^3 cells/second. Analysis of the DNA distributions of L1210 cells on days three, four, six, and seven following implantation of 10^5 cells revealed changes in cycle kinetic patterns concomitant with increased cell density or tumor age. DNA distribution patterns for Lewis lung tumors showed a 2C peak and a cell proliferation pattern extending between the 4C and 8C peaks. Cells sorted electronically from the 2C and 4C-8C contents of the DNA spectrum were identified morphologically as normal and tumor cells, respectively. Tumor cells exhibited elevated protein distributions compared to normal cells; however, tumor cells were quite heterogeneous in cell size and morphology. Analysis of cellular DNA and protein in various tumor systems permits characterization

of the growth kinetics at various stages of tumor development, and such information is of predictive value in chemotherapeutic regimen scheduling. Cell analysis and sorting provide a method for detection and visual inspection of tumor cell populations.

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TABLE I

Cell-Cycle Distribution of L1210 Ascites Cells on Various Days following Inoculation[a]

Day	Percentage of cells		
	G_1	S	G_2 + M
2	71.0	15.8	13.2
3	40.6	50.0	9.4
4	32.9	59.2	7.9
5	30.1	63.7	6.2
6	45.1	46.7	8.2
7	38.8	46.9	14.3
8	48.7	36.6	14.7

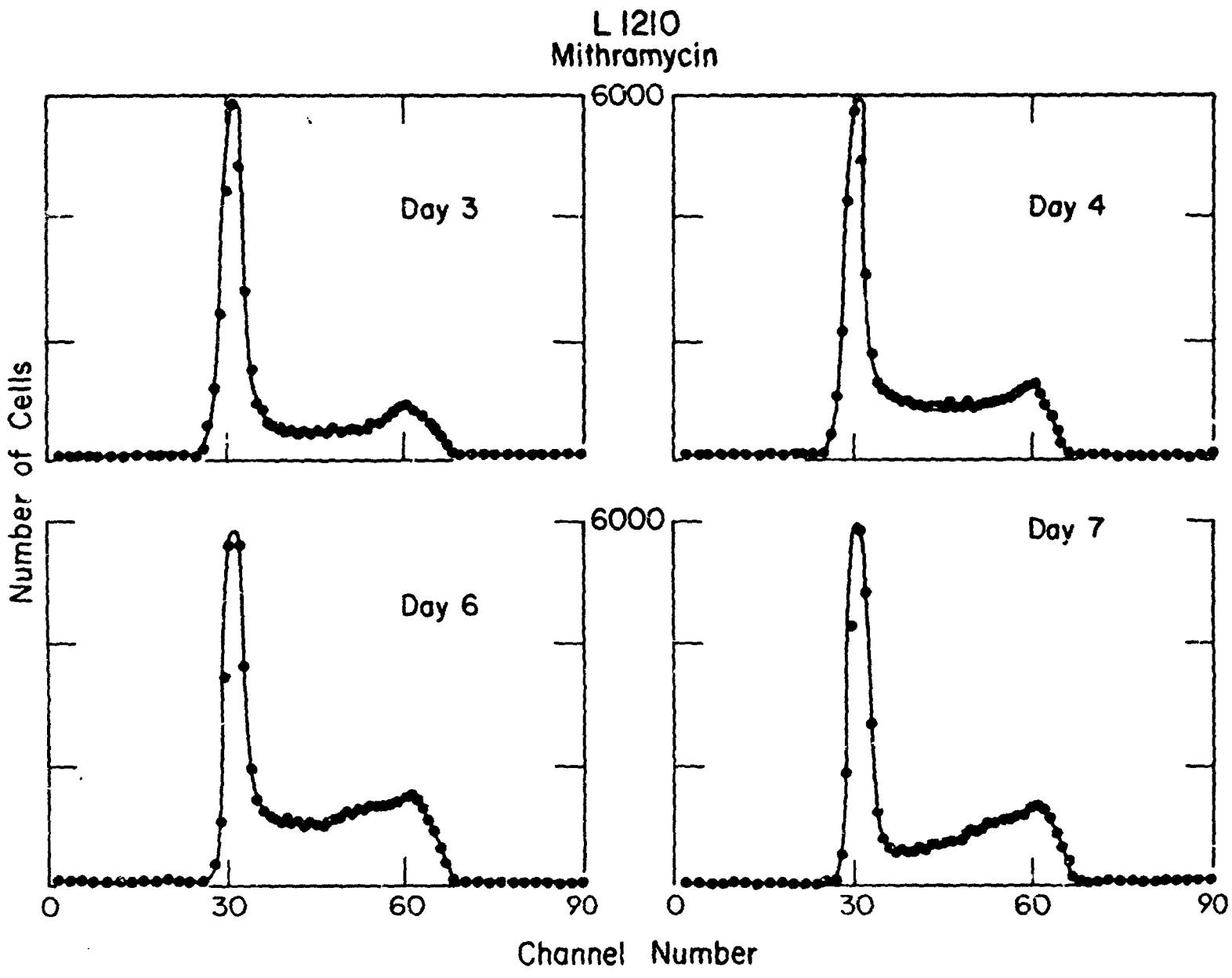
[a]Represents averages of at least three samples.

Fig. 1. DNA content distributions of mithramycin-stained L1210 ascites cells grown in DBA/2 mice and harvested on days three, four, six, and seven.

Fig. 2. Single-parameter and dual-parameter analysis of DNA and protein in PI-FITC stained L1210 ascites cells.

Fig. 3. Computer-generated contour profiles of DNA and protein content of PI-FITC stained L1210 ascites cells grown in DBA/2 mice and harvested on days three, four, six, and seven. The contour density lines are at 20, 100, 200, 500, and 700 cells.

Fig. 4. Computer-generated contour profiles of DNA and protein content of PI-FITC stained primary and lung metastases of the Lewis lung carcinoma. The contour lines are at 50, 100, 150, 200, 350, and 500 cells.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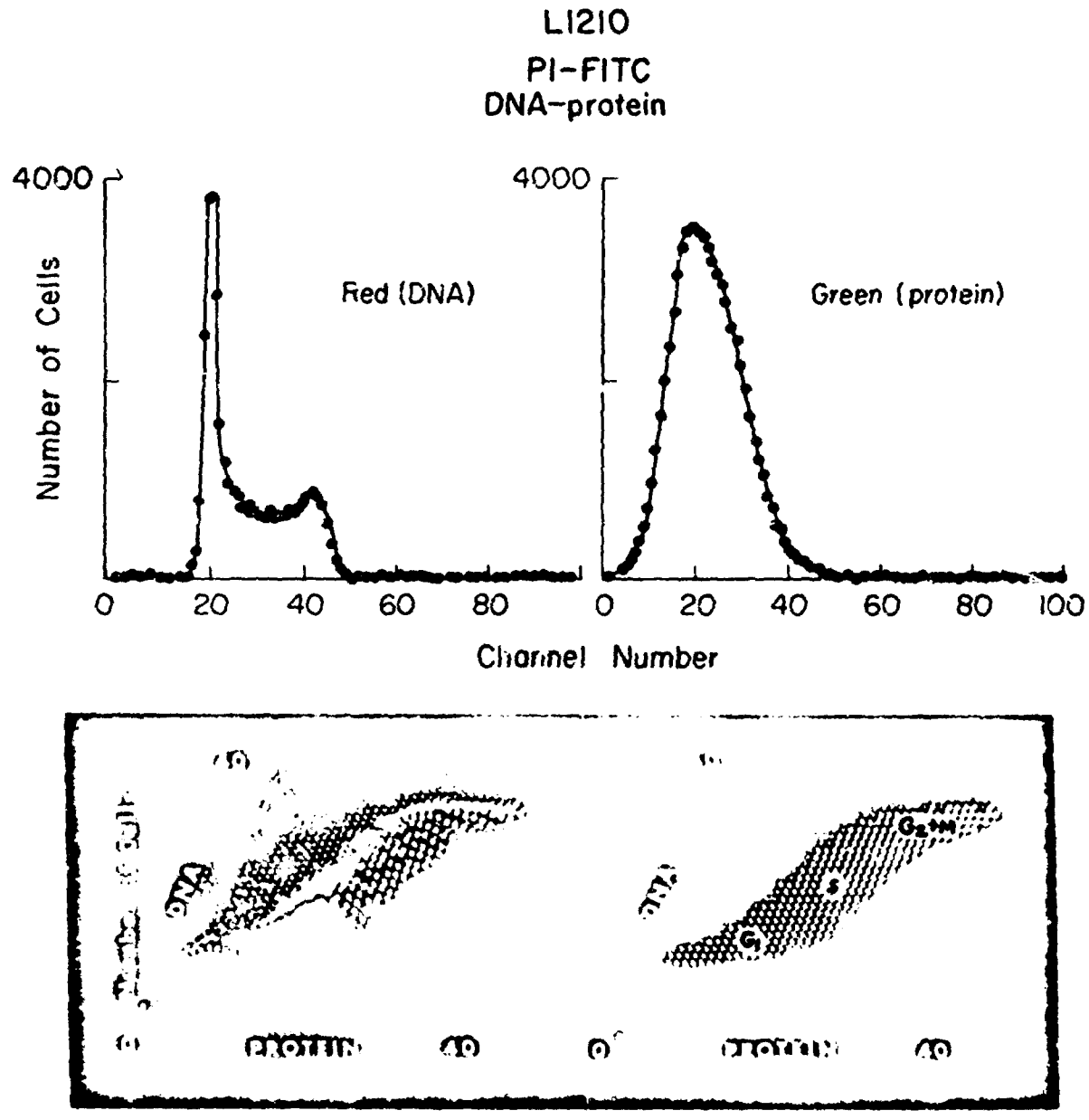
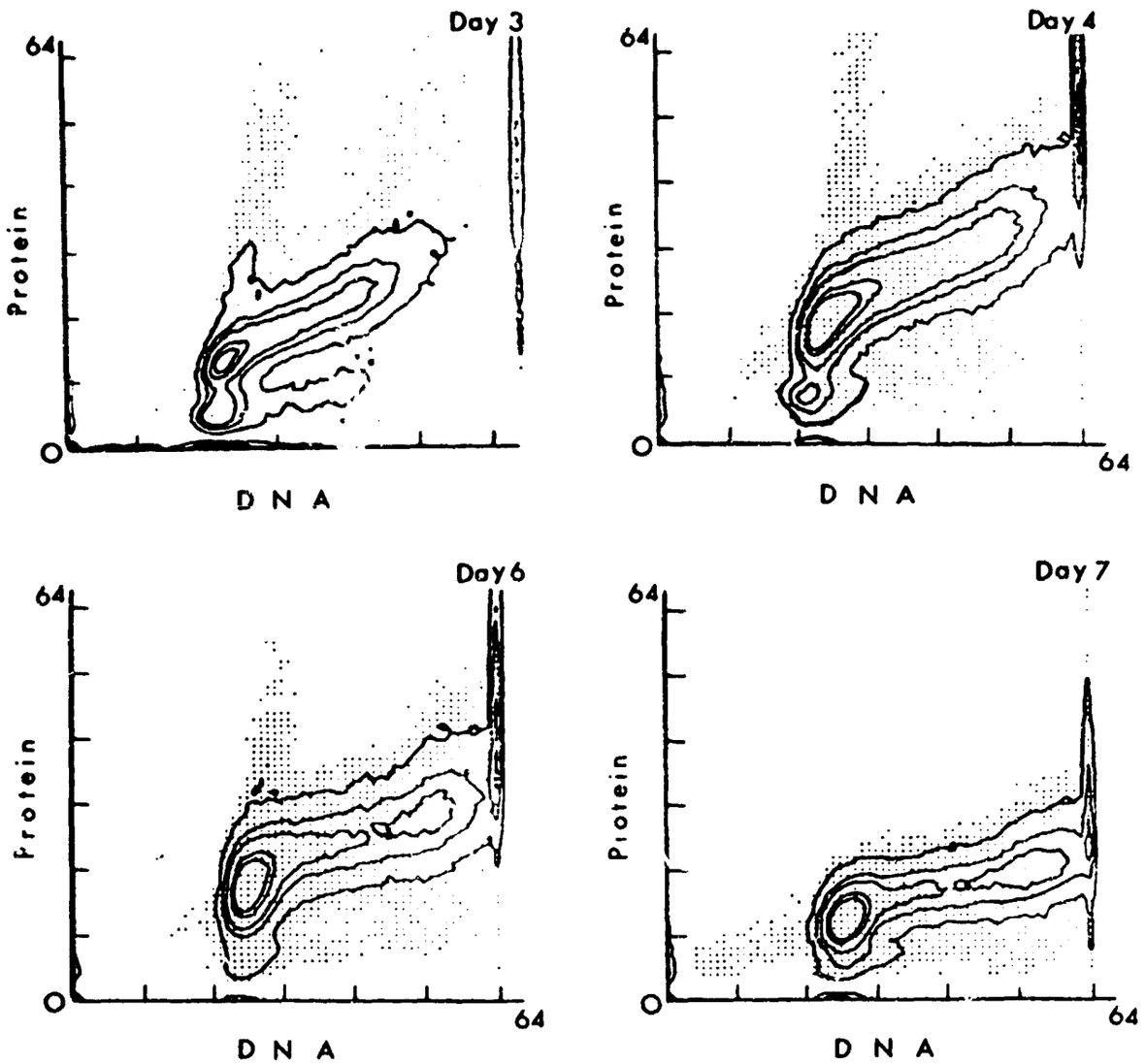
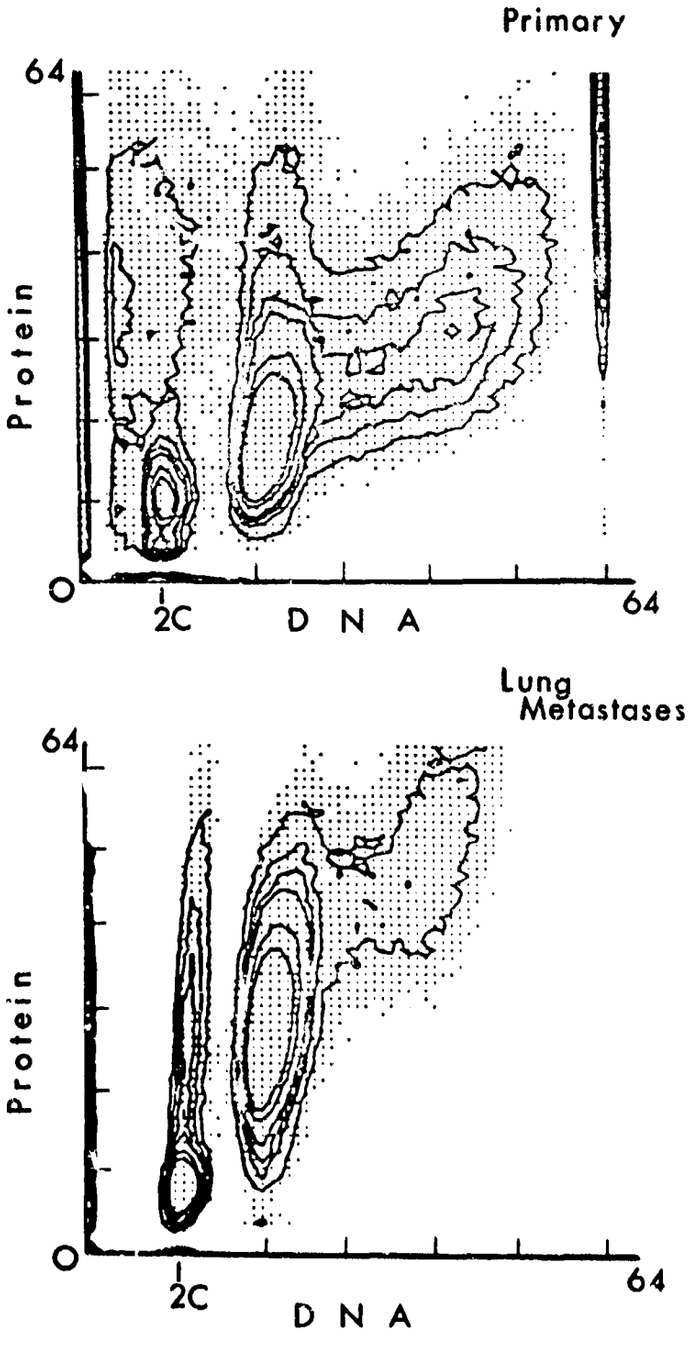
Day 3
Day 4
Day 6
Day 7
Number of Cells
0
30
60
90
Channel Number


L1210
PI-FITC
DNA-protein
4000
Number of Cells
Red (DNA)
4000
Green (protein)
0
20
40
60
80
0
20
40
60
80
100
Channel Number

L1210 Ascites



Lewis Lung Carcinoma



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